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Chlamydiae and Atherosclerosis: Can Psittacine Cases Support the Link?

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SUMMARY. Atherosclerosis is a common disease in pet birds, particularly in psittacines. Little is known about the role of risk factors predisposing birds to this disease. In our study we tried to detect chlamydiae in formalin-fixed and paraffin-embedded atherosclerotic tissue from 103 pet birds in order to clarify their role in atherosclerosis. Methods used were PCR, sequencing and immunohistochemistry. Histopathologic examination served to classify the extent of atherosclerotic lesions. In the PCR, 4 (3.9%) of 103 cases – all of them with advanced stages of atherosclerosis - were positive. Subsequent sequence analysis revealed high identities (94-100%) with *Chlamydophila psittaci* in 3 cases. Interestingly, 2 of these birds came from *Chlamydophila psittaci*-infected populations. Due to the low incidence (3.9%), the occurrence only in advanced stages and the association with *Chlamydophila psittaci*-infected avian populations a causal relationship between chlamydiae and atherosclerosis in pet birds is rather improbable.

Key words: *Chlamydia*, atherosclerosis, pet bird, PCR, immunohistochemistry

Abbreviations: *C.* = *Chlamydia*; *Cp.* = *Chlamydophila*; ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry; PCR = polymerase chain reaction

INTRODUCTION

Atherosclerosis is a slow and progressive disease that usually affects the large and medium sized arteries. As a result of thickening of the artery wall the diameter of the lumen decreases and the blood flow is reduced. In severe cases ischemia in the area receiving blood from the artery occurs.

Atherosclerosis is a principal cause of morbidity and mortality in humans and occurs spontaneously in other mammals and in birds (19). In man risk factors for this cardiovascular disease are among others elevated plasma cholesterol level, hypertension, exposure to cigarette smoking, increasing age, obesity and diabetes mellitus. There are also several studies that have addressed a possible role of infectious agents, such as viruses and bacteria, in atherogenesis (9, 14).

In humans, *Chlamydophila pneumoniae* has been most strongly associated with cardiovascular diseases. Some authors conclude that *Cp. pneumoniae* is a possible etiological agent of atherosclerosis, while others deny it (2, 16, 17).

In birds, the herpesvirus of Marek`s disease was shown to induce atherosclerosis in chickens (12, 13).

Atherosclerosis is also a common disease in birds, especially in orders such as Anseriformes (swan, geese, duck), Columbiformes (pigeons, doves) and Psittaciformes (parrots, parakeets). Within the last order the parrots are most susceptible to atherosclerosis, e.g. the African grey parrot and the amazons (3, 4, 19). Atherosclerotic changes are mainly located in the thoracic part of the aorta and the brachiocephalic arteries. Clinical signs resulting from atherosclerosis are rare. Thus atherosclerosis is usually observed as an unexpected lesion at necropsy (4, 19). The role of risk factors predisposing birds to atherosclerosis is unknown, but since the lesions in birds closely resemble those in man, risk factors in humans may extend to birds (4). The intention of our retrospective study was to detect chlamydiae in formalin-fixed atherosclerotic tissue from birds, in order to clarify their role in atherosclerosis.

MATERIAL AND METHODS

Selection of cases

Formalin(4%)-fixed and paraffin-embedded tissue specimens from 103 pet birds necropsied between 1991 and 2005 at the National Reference Centre for Poultry and Rabbit Diseases (NRGK), University of Zurich, showing macroscopic atherosclerosis were investigated. Tissue specimens were collected from the heart and/or the main-stem vessels.

Histopathology

Histologic sections of all selected cases were stained with the hematoxylin and eosin (HE) technique and reviewed histologically to verify the presence of atherosclerosis. The extent of atherosclerosis was classified in 4 grades (Fig. 3): grade 1 = slight atherosclerosis with fragmentation of the elastic fibres and increase of the extracellular matrix in the intima and media, grade 2 = moderate atherosclerosis with additional accumulation of lipids in the intima, grade 3 = development of plaques consisting of lipids and cholesterol clefts, sporadic cartilage and calcification, grade 4 = severe atherosclerosis with intense and irregular thickening of the arterial wall with plaques containing more cartilage and calcification than those of grade 3.

DNA extraction for PCR screening

From each paraffin block 30 µm sections were cut and placed in sterile microcentrifuge tubes. Then 1ml xylene was added to remove the paraffin. After centrifugation at 15,000rpm for 5 min, the tissue pellet was extracted twice with absolute ethanol followed by centrifugation (15,000rpm; 5 min) to remove remaining xylene, dried at 42 C for 30 min and resuspended in 200µl extraction buffer (containing 0.05% Tween 20, 1mM EDTA, 50mM Tris-HCl pH 8.5). After addition of 2µl 0.2mg/ml proteinase K, samples were incubated at 55 C over night. Then proteinase K was inactivated by heating for 10 min at 100 C. DNA was purified with the phenol-chloroform-isoamylethanol method according to a standard protocol (Sambrook et al, 1989). Briefly samples were mixed twice with 200µl phenol with following incubation for 20

min on ice and centrifugation at 10,000rpm for 5 min. Subsequently the water phase was transferred to a clean tube and treated twice with IAC (chloroform-isoamylethanol 24:1) with following centrifugation at 10,000rpm for 5 min. Afterward DNA was precipitated with 20µl sodium acetate and 400µl absolute ethanol for 30 min at –80 C. Next samples were centrifuged at 13,000rpm for 30 min at 4 C and DNA was washed with 200µl ethanol 70%. After another centrifugation for 15 min the pellet was air-dried and resuspended in 20µl Nuclease-free water (Promega,WI USA).

PCR detection of chlamydial DNA

DNA extracted from formalin-fixed and paraffin-embedded tissue samples was screened for the presence of chlamydial DNA. Due to possible DNA degradation the primer pair of 23SQAPF2 (5' –GAACCTGAAACCA(AG)TAGC-3') and 23SAPR (5'-CTGGCTCATCATGCAAAAGG-3') was applied to amplify a small 92-bp fragment of the 23S rRNA gene. PCR was done in 25µl reaction mixture volumes consisting of 20 pmol of each primer, 12.5µl of HotStarTaq Master Mix from Qiagen (containing HotStarTaq DNA polymerase, PCR buffer with 1.5mM $MgCl_2$ and 200µM each dNTP) and 1µl of each DNA template. The thermal cycling conditions were 95 C for 15 min, 45 cycles of denaturation at 94 C for 30 s, primer annealing at 50 C for 30 s, and elongation at 72 C for 30 s, and a final elongation at 72 C for 5 min (24). Each run contained a negative control with Nuclease-free water substituting the template DNA, and a positive control obtained from formalin-fixed and paraffin-embedded tissues containing chlamydial DNA (either from birds or gnotobiotics piglets). 10µl of each PCR product were analysed by electrophoresis on an ethidium bromide-stained 2% agarose gel (Agarose Standard, Eurobio) in the presence of a 100bp ladder. PCR bands on the gel were visualized by UV illumination with a computer-aided bioimage system (Bio-Capt, LTF, Wasserburg, Germany).

Sequencing

Of each PCR product 60µl was electrophoresed on an ethidium bromide-stained 2% agarose gel (Agarose Standard, Eurobio). Desired bands of amplicons were cut out under UV illumination and DNA was extracted using the MinElute Gel Extraction Kit from Qiagen. Samples were sent to Microsynth AG (Balgach, Switzerland) for direct sequencing. The obtained sequences were analyzed through BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Immunohistochemistry

Paraffin sections showing a suspect band in the PCR were processed for immunohistochemistry by using mouse monoclonal antibodies against chlamydial lipopolysaccharide (clone ACI-P) and Dako EnVision anti-mouse (K 4001). First sections were pretreated by microwave heating for 20 min in citrate buffer (Dako S2031) for antigen retrieval. Then they were incubated with the primary antibody at a working dilution of 1:200 (Dako S2022) for 60 min at room temperature. After incubation for 30 min with the second antibody at room temperature, sections were developed in peroxidase/3-amino-g-ethylcarbazole (AEC) substrate solution for 10 min at room temperature, counterstained with hematoxylin and coverslipped. From each section a negative control was performed using the antibody diluent (Dako S2022) without the primary antibody. As a positive control intestinal tissues from gnotobiotic piglets experimentally infected with *Chlamydia suis* were used.

RESULTS

Selection of cases

The 103 birds consisted of the following avian orders: 92 Psittaciformes, 3 Passeriformes, 2 Anseriformes, 2 Ciconiiformes and 4 birds from other avian orders. In the order Psittaciformes, African grey parrots (n=31) and amazons (n=21) had the highest incidences, with 30.3% respectively 20.4% (Figure 2). The age of the birds ranged from 2 to 78 years (Table 1).

Histopathology

Histopathologic examination revealed in all cases slight to severe atherosclerosis. 15 of the cases were classified grade 1, 20 were rated grade 2, 24 were classified grade 3, and in 40 cases a severe atherosclerosis with grade 4 was observed. No relation between age of bird and degree of atherosclerosis was found (Fig. 1), but there was an association between species and severity of atherosclerosis (Fig.2).

PCR

Of all 103 samples tested, 22 (21.3%) were PCR suspect, showing a band at 50 – 200bp. For further confirmation 30 µm sections from these 22 specimens were cut again and retested with the same method and in the same run, using more positive controls. A strong positive band at 92bp was seen in 4 specimens, resulting in a total rate of 3.9% (n=103). Positive and negative controls reacted appropriately.

Sequencing

Online BLAST searches revealed for all 4 PCR-positive samples applied for direct sequencing similarities with sequences of the genus *Chlamydophila*. In 3 cases high identities

(94-100%) with *Cp. psittaci* were found, whereas in one case no further specification was possible. Results of the PCR positive cases are summarized in Table 2.

Immunohistochemistry

A positive staining was seen in 9 of the 22 (40.9%) PCR-suspect sections prepared for immunohistochemistry. Granules with positive staining were found mainly at locations of the vessel wall, where the atherosclerotic alterations were severe. In the extracellular matrix as well as in the cytoplasm of macrophages and also of smooth muscle cells immunopositive signals were observed.

In negative control sections immunopositive staining was found at the same locations as in the 9 sample sections.

DISCUSSION

Chlamydiae are unique intracellular pathogens with a biphasic developmental cycle consisting of the infectious elementary body (EB) and the non-infectious reticulate body (RB) (10). Until 1999 the order contained only one genus, *Chlamydia*, with 4 recognized species, namely *C. psittaci*, *C.trachomatis*, *C. pneumoniae* and *C. pecorum* (11). Recently the order has been reclassified on the basis of taxonomic analysis of the 16S and 23S rRNA genes. Three new families, Parachlamydiaceae, Simkaniaceae and Waddliaceae were proposed, while the family Chlamydiaceae was divided into two genera, *Chlamydia* and *Chlamydophila*, with a total of nine species (11). Chlamydiae cause a variety of diseases in different animal species including humans, birds, koalas, cats, pigs, cattle, sheep and goats (10).

Chlamydophila pneumoniae was first described in humans in 1986. It is a common cause of upper respiratory tract infections and accounts for up to 10% of community acquired pneumonia (15). Recently it has been associated with several chronic diseases such as Alzheimer's disease and atherosclerosis. The first report that discussed a possible connection between *Cp. pneumoniae* and atherosclerosis was a serologic study performed in Finland in 1988 (22). Seroepidemiologic studies were followed by studies in which the organism was identified in atherosclerotic tissue by electron microscopy, immunocytic chemical staining and PCR (5, 6, 8, 23). However, the results of those studies are inconsistently reporting a large variability in detection rates (6). Therefore the real prevalence of *Cp. pneumoniae* within atherosclerotic lesions and its role in atherogenesis is still a matter of controversial debate in human medicine.

Atherosclerosis in birds - mainly in parrots - seems to be more prevalent and severe than in any species of mammals, except for men (4). Postmortem findings in birds examined between 1991 and 1997 revealed an incidence for atherosclerosis of 7% for the psittacines, whereas only 0.6% of the passerines showed this disease. Of the atherosclerotic psittacines, 79% were parrots, with the highest incidences for the African grey parrot (35%) and the amazons (22%) (1). In contrast to man, little is known about risk factors of atherosclerosis in birds. Age, genetics, plasma cholesterol levels, diet, inactivity, social stress and obesity were discussed in parrots (4).

Cp. pneumoniae has yet never been found in birds. In a recent study from Sudler et al, all fifty-one chlamydial isolates from birds collected in Switzerland were classified as *Cp. psittaci* (25). On the other hand *Cp. psittaci* is widespread in avian populations, where it causes disease with varying morbidity and mortality (21).

In this study, we investigated the presence of chlamydiae in vascular tissues from 103 pet birds with atherosclerosis. The birds consisted mainly of parrots (89.3%) with the highest incidences for African grey parrots and amazons, as described in other reports (3, 4). Atherosclerosis was often an ancillary finding. However atherosclerosis was the only disorder found in the majority of cases classified as grade 4. Histologic classification revealed no association between age of the birds and degree of atherosclerosis (Fig 1). This is in contrast to a study showing that severity of atherosclerosis in parrots increased with age (3). However, in our study smaller parrots with lower expectation of life – like cockatiels and lovebirds - with severe atherosclerosis were also investigated. On the other hand, none of the macaws that can reach high ages, showed severe atherosclerosis, as reported by Bavelaar and Beynen (3).

DNA degradation in formalin-fixed and paraffin-embedded sections was one of the problems in our study - the older the samples, the larger the chance of encountering a fragmented DNA (21). Our attempts with primer pair 16SF2 (5'-CCGCCCGTCACATCATGG-3') and 23R (5'-TACTAAGATGTTTCAGTTC-3') to amplify a 585bp-product of the 16S/23S rRNA intergenic spacer region of chlamydiae were not successful (unpublished data). Therefore it was important to design a PCR assay that amplified a short DNA fragment for the detection of chlamydiae in our samples. Only 4 of 103 birds – two amazons, one budgerigar and one jacana - tested with a 23s rRNA PCR that amplifies a 92bp fragment were positive. With online BLAST searches a high similarity to *Cp. psittaci* for the two amazons and the jacana was found. No further identification was possible with the budgerigar sample. This may be due to a new species or a species, which was not described as yet.

Immunohistochemistry was not to our satisfaction. None of the 9 IHC-positive sections could be confirmed by repeated PCR run. Furthermore, sections from the 4 PCR positive cases were negative in IHC. Poor correlation between PCR and IHC in atherosclerotic tissue has been reported in other studies (6, 16). Of major concern are non-specific staining reactions of the chlamydial antibodies with cell components in the atherosclerotic plaque. Hoymans et al. could show a high affinity of the chlamydial antibodies to oxidized low-density lipoproteins in atherosclerotic lesions, a phenomenon of molecular mimicry that leads to false-positive results (16). Another explanation could be that chlamydia antigens rather than viable bacteria persist in the specimens, as described by Meijer et al. (20). However our study confirms the observation of Hoymans et al. as even negative control sections demonstrated non-specific staining reactions. Furthermore false negative IHC-results could be ascribed to the fact that the small sections tested did not contain any chlamydial antigen.

Interestingly, the two amazons sequenced positive for *Cp. psittaci* came from *Cp. psittaci*-infected populations and had been tested for chlamydia antigen at post mortem with the Chlamydia-antigen ELISA (IDEIA™ Chlamydia ref K6002, DakoCytomation, Denmark). It was performed on liver, spleen and cloacal samples with a negative result in both birds. The PCR, which is more sensitive, suggests that the level of chlamydial antigen was too low to be detected in the ELISA.

An etiological role of chlamydiae can't be confirmed by our study. If chlamydiae cause atherosclerosis in birds, their presence would be expected to precede that of disease. One hypothesis states chlamydiae to act as focal point in atherosclerosis. After initiation of the disease, they disappear from the lesions. In this case one would expect to find chlamydiae with a higher prevalence in slight atherosclerotic lesions rather than in the severe ones. Furthermore it is still possible that after initiating the disease process the organism has disapperared and thus is not detectable when atherosclerosis develops. However experimental infections with different *Cp. psittaci* strains in birds showed, that no signs of atherosclerosis were found at necropsy, even 210 days post infectionem (7, 18). In another hypothesis chlamydiae initiate atherosclerosis and persist in vascular tissue. Thus their prevalence would be similar in every grade of atherosclerosis. If chlamydiae play no role in atherosclerosis, but prefer the altered vascular tissue as a favorable environment for persistence or growth, one would expect to detect them mainly in lesions of grade 3 or 4. In our study chlamydiae were only found in older lesions (grade 3 or grade 4), suggesting no role for them as initiators of atherosclerosis.

In conclusion, we could detect chlamydiae in atherosclerotic tissue from 4 of 103 birds. The low incidence (3.9%), the occurrence only in advanced stages of atherosclerosis and the association with Cp. psittaci-infected avian populations, leads us to the conclusion, that there is probably no association between chlamydiae and atherosclerosis in pet birds.

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Table 1. Avian orders and age of collected birds

Avian order	Total	Age (years) 1 to 5	6 to 10	11 to 20	21 to 30	> 30	Unknown
Psittaciformes	92	10	12	15	9	10	36
Passeriformes	3	1	1	0	0	0	1
Anseriformes	2	1	0	0	0	0	1
Ciconiiformes	2	0	0	0	0	0	2
Others	4	0	1	1	0	0	2
Total	103	12	14	16	9	10	42

Table 2. Results of histopathology, immunohistochemistry (IHC) and sequence analysis of the 4 PCR positive cases.

Bird	Grade of atherosclerosis	IHC	PCR	Sequencing
Amazon	4	-	+	*Cp. psittaci*
Budgerigar	ne	-	+	*Cp. species*
Amazon	3	-	+	*Cp. psittaci*
Jacana	4	-	+	*Cp. psittaci*

ne: not evaluated

Fig. 1. Distribution of the degree of atherosclerosis among the different age groups (only 56 psittacines with known age are represented)

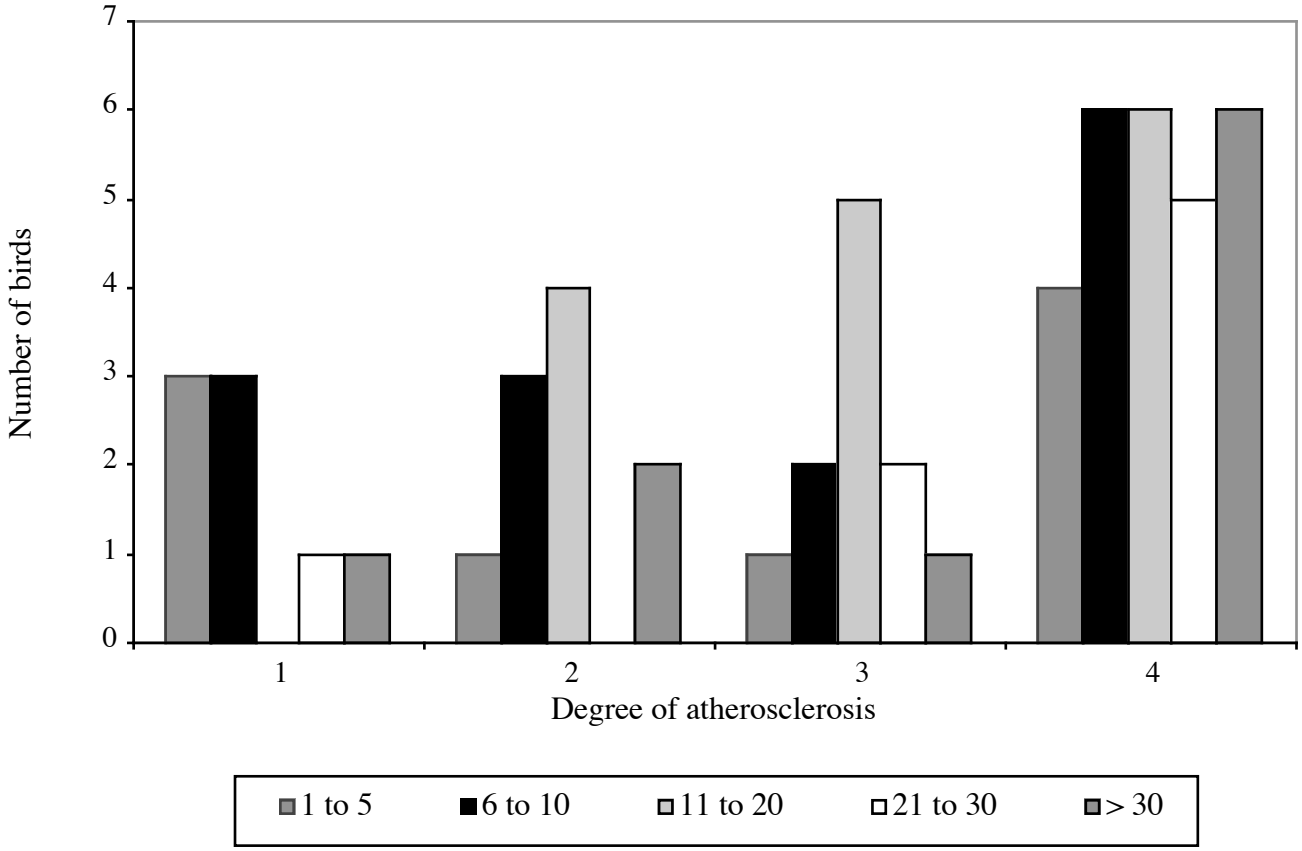
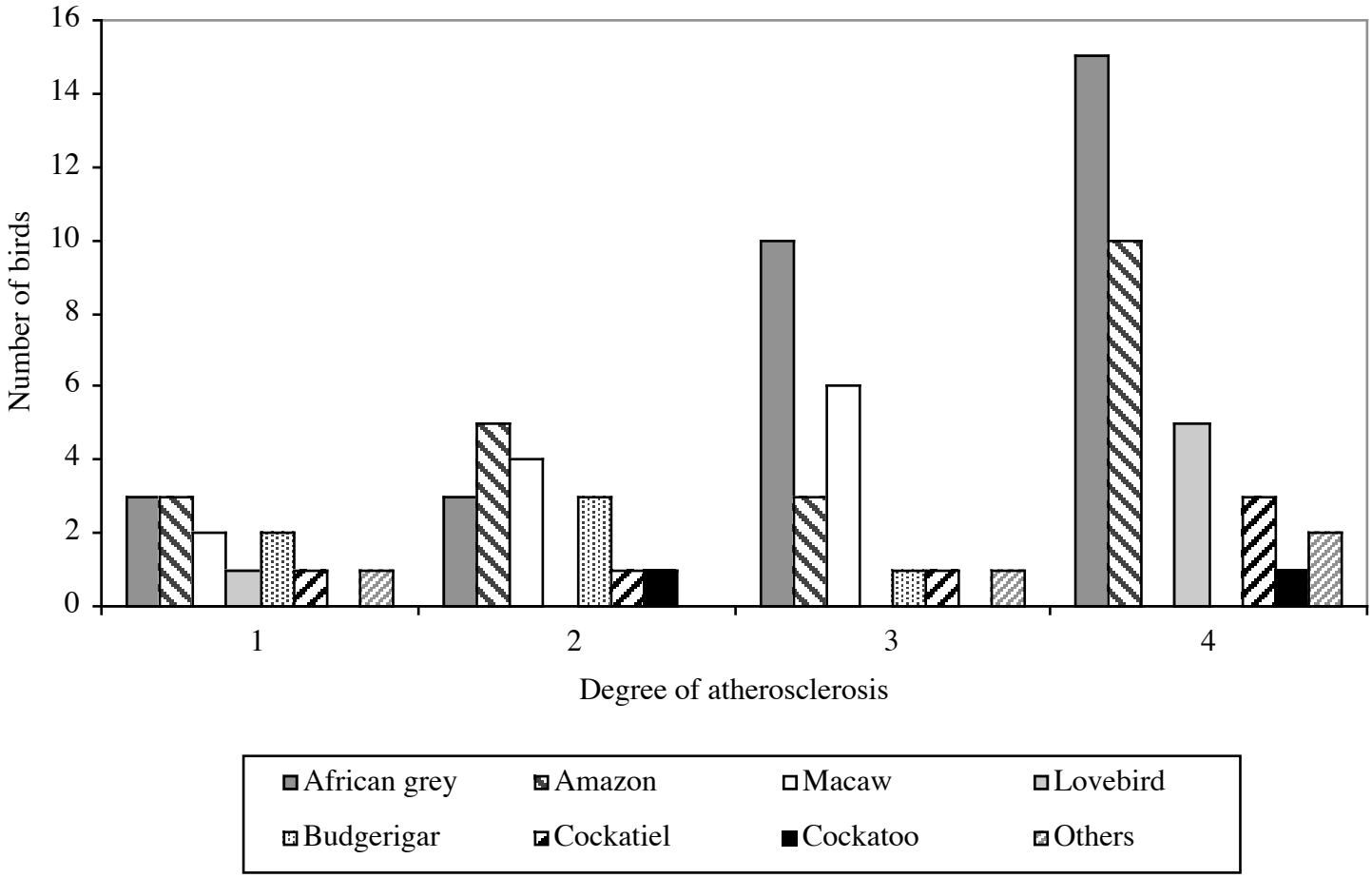


Fig. 2. Distribution of the degree of atherosclerosis among the different species



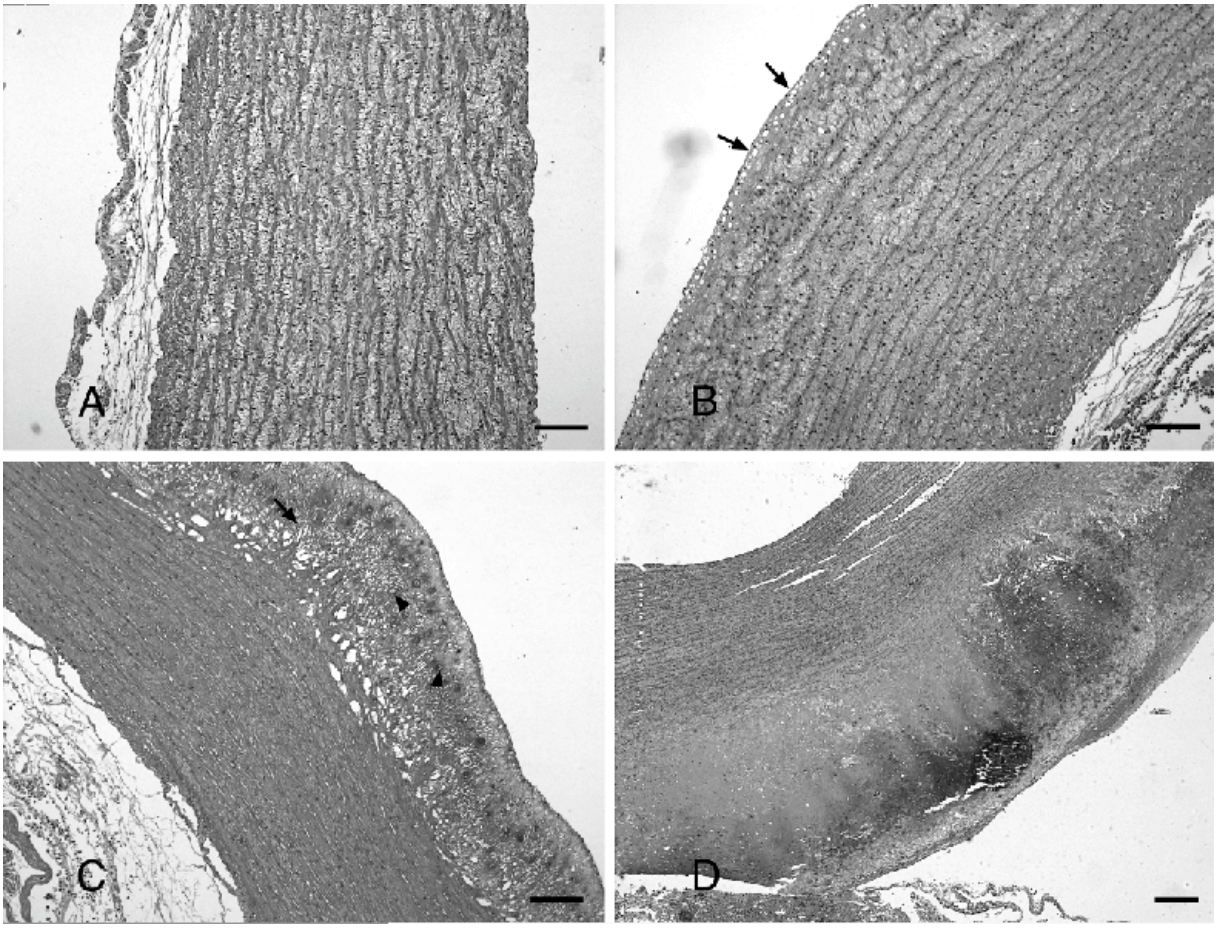


Fig. 3. The extent of atherosclerosis in 4 grades. (A) Grade 1 = slight atherosclerosis with fragmentation of the elastic fibres and increase of the extracellular matrix in the intima and media. Bar = 100 μm. (B) Grade 2 = moderate atherosclerosis with additional accumulation of lipid vacuoles (arrows) in the intima. Bar = 100 μm. (C) Grade 3 = development of plaques consisting of lipids, cholesterol clefts (arrow), fibrous connective tissue and chondrocytes (arrowheads). Bar = 100 μm. (D) Grade 4 = severe atherosclerosis. Bar = 200 μm.

Lebenslauf

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